NORTHERN ILLINOIS UNIVERSITY

Regulation and Role of Calcium in Pancreatic Exocytosis

A Thesis Submitted to the

University Honors Program

In Partial Fulfillment of the

Requirements of the Baccalaureate Degree

With University Honors

Department of Biological Sciences

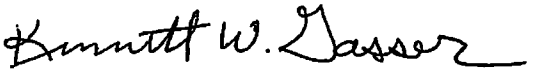
by

Stacy Jeanne Hanc

DeKalb, Illinois

May 9, 1998

Student Name: Stacy Jeanne Hanc

Approved by: Kenneth W. Gasser 

Department of: Biological Sciences

Date: May 9, 1998

HONORS THESIS ABSTRACT

THESIS SUBMISSION FORM

AUTHOR: Stacy Jeanne Hanc

THESIS TITLE: Regulation and Role of Calcium in Pancreatic Exocytosis

ADVISOR: Kenneth W. Gasser ADVISOR'S DEPT: Biological Sciences

DISCIPLINE: Cell Biology YEAR: 1998

PAGE LENGTH: 54 BIBLIOGRAPHY: pp. 50-54

PUBLISHED: No

COPIES AVAILABLE: Hard Copy

ABSTRACT: The pancreas is an organ designed to synthesize, package, and release enzymes required by the digestive system. These enzymes are packaged in secretory vesicles and are released by exocytosis following stimulation of the pancreatic acinar cells by gastrointestinal hormones. A recognized aspect of this signal is an increase in the concentration of intracellular Ca^{2+}. Previous work has demonstrated that the increase occurs preferentially in the apical pole of the cell in the region of the secretory vesicles prior to the onset of exocytosis. Secretory vesicles were determined to contain significant amounts of Ca^{2+} by FURA-2 fluorescence. Purified secretory vesicle membranes were subjected to SDS-PAGE and immunoblotted with antibodies to a consensus region of ryanodine and IP_3 receptors. The results show that the pancreatic secretory vesicles express both of these Ca^{2+} release pathways and is then consistent with the vesicles acting as a physiologically relevant Ca^{2+} store. Efflux of Ca^{2+} through these release pathways will require the participation of a counterion. Theoretically, K^+ influx or Cl^- efflux could serve as the requisite counterion to Ca^{2+} efflux. Experimental conditions that promote a Cl^- flux increased the rate of secretory vesicle swelling. This swelling could be prevented by treatment with the Ca^{2+} channel blockers $NiCl_2$, $LaCl_3$, and ruthenium red. Taken together, the results indicate that secretory vesicles, under the in vitro assay conditions, release Ca^{2+} preferentially through a ryanodine receptor Ca^{2+} channel and that the rate of release was limited by Cl- transport. The contribution of these pathways to exocytosis was subsequently determined through an analysis of secretory vesicle fusion with apical membranes in vitro. However, blockage of the Ca^{2+} efflux pathway by ruthenium red or $LaCl_3$, significantly reduced the rate of membrane fusion. These results suggest a model whereby Cl^- dependent Ca^{2+} efflux from the luminal vesicle environment is a requirement for exocytosis and membrane fusion.

ACKNOWLEDGEMENTS

I would like to thank Professor Kenneth W. Gasser for all of the guidance, faith, and time he invested in me. His dedication to research and science will always be an inspiration to me. Financial support for the research was provided by a NIH grant to Dr. Gasser. I appreciate the time Chris Johnson spent helping me get accustomed to the lab in my first months doing research. My project would not have been as successful or enjoyable without the help of my fellow lab partner/classmate/friend, Heath McDonald. I thank Kim Ackert for reminding me there is a life outside the lab. I am also grateful to Tom for his extreme patience, understanding, and his continual support for all my aspirations. I am forever indebted to my Mom and Dad, who both have helped me to deal with the anxiety associated with hard work, and have taught me how to strive for perfection. And finally, my brother Brian, who always puts a smile on my face and continually impresses me with his abilities and relentless determination. I look at all of his accomplishments and use them as a reminder that I can make my dreams become a reality.

INTRODUCTION

The role of the exocrine pancreas is to synthesize, package, and release digestive enzymes in response to stimulation by gastrointestinal hormones. These enzymes include trypsin, chymotrypsin, carboxypeptidase, amylase, lipases, and ribonucleases to name a few. The typical secretion pathway begins with translation of the appropriate mRNA at the endoplasmic reticulum (ER). These nascent peptides contain an N-terminal ER signal sequence that directs the peptides to the ER lumen cotranslationally. Following release of the translated peptide into the ER lumen, the N-terminal sequence is cleaved by an ER resident peptidase. Proteins destined for secretion (i.e. digestive enzymes) are packaged into vesicles and transported to the golgi apparatus. Within this organelle, the proteins are further modified and directed to the trans golgi network, where they are packaged into secretory vesicles. Proteins are targeted to these secretory vesicles based on an association for secretogranin, a vesicle resident membrane protein. The packaged proteins subsequently precipitate within the vesicle lumen in response to an increase in Ca^{2+} and a decrease in pH (Nicaise et al., 1992). The digestive enzymes are finally released into the pancreatic duct by exocytotic fusion of the secretory vesicle membrane with the apical plasma membrane.

Exocytosis is promoted by a signaling pathway which includes the production of cAMP by adenylyl cyclase and the increase in cytoplasmic Ca^{2+} by release from intracellular stores. These second messengers alter the activity of

kinases and docking proteins that are part of the mechanism controlling the terminal exocytotic event (Edwardson and Marciniak, 1995). In addition to the requisite docking proteins, secretory granule membranes have previously been shown to contain a ClC-2 type Cl^- channel (Gasser and Hopfer, 1990; Carew and Thorn, 1996), an ATP-sensitive K^+ channel (K_{ATP}) (Thevenod et al., 1992; Gasser and Holda, 1993), Cl^-/ HCO_3^- exchange transporters (Gasser et al; 1988), Na^+/H^+ exchange transporters (Anderie and Thevenod, 1996), and a RAB3 g-protein (Gasser et al., 1997; Ohnishi et al., 1996). Previous studies have shown that activation of these electrolyte transport pathways induced vesicle swelling and increased the efficiency of exocytotic membrane fusion (Stahl, 1996). Furthermore, the data were consistent with the premise that the RAB3 g-protein controls the open state of the K_{ATP} channel (Gasser et al., 1997).

The intracellular signaling pathways responsible for regulating these proteins, and ultimately pancreatic exocytosis, are initiated by the binding of a gastrointestinal hormone such as cholecystokinin (CCK). Hormone binding with the plasma membrane CCK receptor causes a conformational change in the receptor that induces the associated Gq g-protein to release GDP and bind GTP. This initiates the liberation of the α-subunit from the g-protein complex, which in turn binds to and allosterically activates phospholipase-C (PL-C). Once activated, PL-C will cleave the membrane phospholipid phosphotidylinositol 4,5-bisphosphate and produce 1,4,5-trisphosphate (IP_3) and diacylglycerol (Berridge, 1993). IP_3 is water soluble and is released into the cytoplasm of the acinar cell where it binds to a receptor on the ER. This ER receptor is also a Ca^{2+} channel,

and IP_3 binding induces this release pathway to open, permitting Ca^{2+} efflux from the intracellular store into the cytoplasm. This increase in cytoplasmic Ca^{2+} is a central feature of secretion induction in all cells including the pancreas, and is required to activate the pathways leading to exocytosis .

In virtually all cells, intracellular Ca^{2+} regulation is a complex process utilizing many different pathways. The ultimate source of Ca^{2+} for all cells is the extracellular fluid where the concentration is approximately 2mM. Many cells utilize this extracellular source of Ca^{2+} almost exclusively, including many types of smooth muscle and cardiac muscle. These are excitable cells and contain voltage gated Ca^{2+} channels at the plasma membrane. Upon depolarization of the plasma membrane (i.e. an action potential), these channels open and permit an influx of Ca^{2+} from the extracellular fluid to the cytoplasm, a prerequisite for muscle contraction. Relaxation requires that the Ca^{2+} concentration decrease to baseline levels, which is accomplished by the plasma membrane Ca^{2+}-ATPase and a Na^{+}/Ca^{2+} exchange. Other excitable cells such as skeletal muscle use a combination of extracellular and intracellular stores of Ca^{2+} to regulate the cytoplasmic concentration. In this unique system, the change in membrane voltage activates a plasma membrane dihydropyridine (DHP) receptor. This receptor alters its confirmation in response to the voltage change and promotes an interaction with a ryanodine receptor (located on the sarcoplasmic reticulum) which is closely juxtaposed to the DHP receptor (located on the plasma membrane) (Rios et al., 1992). This positioning permits an extracellular signal (i.e. voltage change) to be communicated to an intracellular compartment such

as the sarcoplasmic reticulum (SR). The ryanodine receptor, like the previously mentioned IP_3 receptor, is a Ca^{2+} release channel. Once activated by the DHP receptor, the ryanodine receptor will change its conformation and allow the efflux of Ca^{2+}, resulting in an increase in cytoplasmic Ca^{2+} concentration. Due to the Ca^{2+} induced-Ca^{2+} release (CICR) characteristic of the ryanodine receptor, the initial release of Ca^{2+} will in turn stimulate high affinity Ca^{2+} binding sites on other SR ryanodine receptors, causing them to open and release Ca^{2+} as well (Sorrentino, 1995). This further release of Ca^{2+} into the cytoplasm will subsequently induce low affinity Ca^{2+} sites to be bound and cause the channels to close. This mechanism (i.e. opening at low Ca^{2+} concentrations and closing at high Ca^{2+} concentrations) limits the amount of Ca^{2+} that can be released after a rapid positive feedback response (Striggow and Ehrlich, 1996). The SR Ca^{2+} - ATPase will subsequently begin to resequester the Ca^{2+} from the cytoplasm to the store and drive the cytoplasmic concentration back to the baseline resting value.

The ryanodine receptor Ca^{2+} release pathway is a well characterized component in skeletal muscle Ca^{2+} regulatory mechanism. However, more recent studies have shown ryanodine receptors to be found in many tissues beyond skeletal muscle (McPherson and Campbell, 1993). To date, three different subtypes of the ryanodine receptor have been identified associated with either the sarcoplasmic reticulum (SR) or ER Ca^{2+} stores. The type 1 receptor is localized exclusively to excitable tissues, type 2 receptor has been found in heart, stomach, brain, and endothelial cells, and the type 3 receptor has been

found in a variety of non-excitable tissues as well as smooth muscle and brain (Sorrentino, 1995). Different ryanodine receptor Ca^{2+} channel subtypes can be opened in response to an interaction with the DHP receptor, the second messenger cADP-ribose, or by CICR. The open probability of these channels can be further modified in response to changes in the concentration of intracellular ATP and ADP as well as phosphorylation and dephosphorylation of the channel (Valdivia et al., 1997). Therefore, it has become apparent that this Ca^{2+} release pathway is a widely distributed control mechanism for the regulation of intracellular Ca^{2+} and is physiologically significant well beyond the commonly known control of skeletal muscle contraction.

The other common Ca^{2+} store in most nonexcitable cells is under the control of IP_3 and the IP_3 receptor Ca^{2+} channel. Three subtypes of the IP_3 receptor have also been identified, which exhibit differential distributions at both the tissue and subcellular levels. The IP_3 receptor is typically regulated, as previously noted, by the cleavage of the inositol phosphate head group from phosphatidylinositol 4,5-bisphosphate. Cells have the capacity to produce up to 9 different inositol phosphates which in turn have different affinities for the 3 subtypes of the IP_3 receptor (Mikoshiba, 1997). The IP_3 receptor Ca^{2+} channel can be further regulated by the intracellular concentrations of Ca^{2+}, ATP, and ADP as well as the phosphorylation state of the receptor.

IP_3 receptors have also been found in cellular locations other than the ER, suggesting that this pathway may regulate Ca^{2+} flux from multiple stores. The type-3 receptor has been postulated to reside at the plasma membrane of some

cells (Putney, 1997). Furthermore, IP_3 receptors have been found on the nuclear membrane, whereas in cell systems that lack ER (such as sperm), they were found on acrosomal secretory vesicle membranes (Walensky and Snyder, 1995).

These release pathways are responsible for increasing the concentration of intracellular Ca^{2+}. However, physiological control necessitates that downregulation must also occur, and there is an entirely different set of pathways and mechanisms responsible for decreasing the concentration of cytoplasmic Ca^{2+}. In order to achieve Ca^{2+} efflux from the cell, an energy source is required due to the substantial electrochemical gradient that exists across the membrane. All cells possess a plasma membrane Ca^{2+} -ATPase which is responsible for pumping Ca^{2+} from the cytoplasm to the extracellular environment. This Ca^{2+} pump utilizes the energy of ATP hydrolysis to power the movement of Ca^{2+} against its gradient. In addition, a separate Ca^{2+}-ATPase with different pharmacological properties, is located on the ER or SR to resequester Ca^{2+} from the cytoplasm to the intracellular store. Another mechanism existing on the plasma membrane is the Na^+/Ca^{2+} exchange transporter. This exchange system uses the inwardly directed Na^+ electrochemical gradient at a stoichiometry of $3Na^+ : 1Ca^{2+}$ as an energy source to drive Ca^{2+} efflux out of the cell. Through the combined efforts of these export pathways, the resting cytoplasmic Ca^{2+} concentration can be reduced to approximately 0.1μm. This level of resting cytoplasmic Ca^{2+} is insufficient to activate most Ca^{2+}-dependent biochemical pathways.

In order to prevent further release of Ca^{2+}, the intracellular signal for Ca^{2+} release must also decrease. This mechanism involves the activity of kinases and phosphatases which alter the phosphorylation state of the inositol sugar and decrease its affinity for the receptor. The inositol head group is subsequently reesterified to diacylglycerol, completing the phospholipid metabolic cycle. Furthermore, PL-C, the enzyme responsible for liberating the IP_3, is downregulated and inactivated when the α-subunit of the associated g-protein hydrolyzes the GTP.

Nonexcitable cells rely heavily on Ca^{2+} release from intracellular stores and a fraction of that Ca^{2+} is always lost to the extracellular environment due to the active plasma membrane efflux pathways described above. Therefore, maintaining the requisite concentration in these stores requires Ca^{2+} uptake from the extracellular environment. The capacitative Ca^{2+} entry pathway at the plasma membrane, is a unique transport system that is activated in response to depletion of Ca^{2+} within the store. By this mechanism, a Ca^{2+} sensor at the intracellular store can communicate the relative Ca^{2+} concentration to the plasma membrane influx pathway. When the stores become depleted, the plasma membrane influx pathway opens, allowing influx of Ca^{2+} to be used to refill the store (Putney, 1997). Pancreatic acinar cells, as with all nonexcitable cells that have been investigated, rely on this unique transport pathway to maintain long-term Ca^{2+} homeostasis (Bahnson et al., 1993).

Ca^{2+} regulation in the pancreas has been studied extensively and has lead to a thorough understanding of many of the regulatory features. Previous studies

have shown that upon hormonal stimulation, Ca^{2+} concentration increases at the apical pole of the pancreatic acinar cell prior to the basal pole (Lee et al., 1997). Histological studies have shown that the apical pole contains predominantly mature secretory vesicles whereas the basal pole contains the nucleus and ER. Based on the temporal asymmetry of intracellular Ca^{2+} concentration following agonist stimulation, other release pathways or Ca^{2+} stores (either intracellular or extracellular) may be responsible for the observed pattern of Ca^{2+} release.

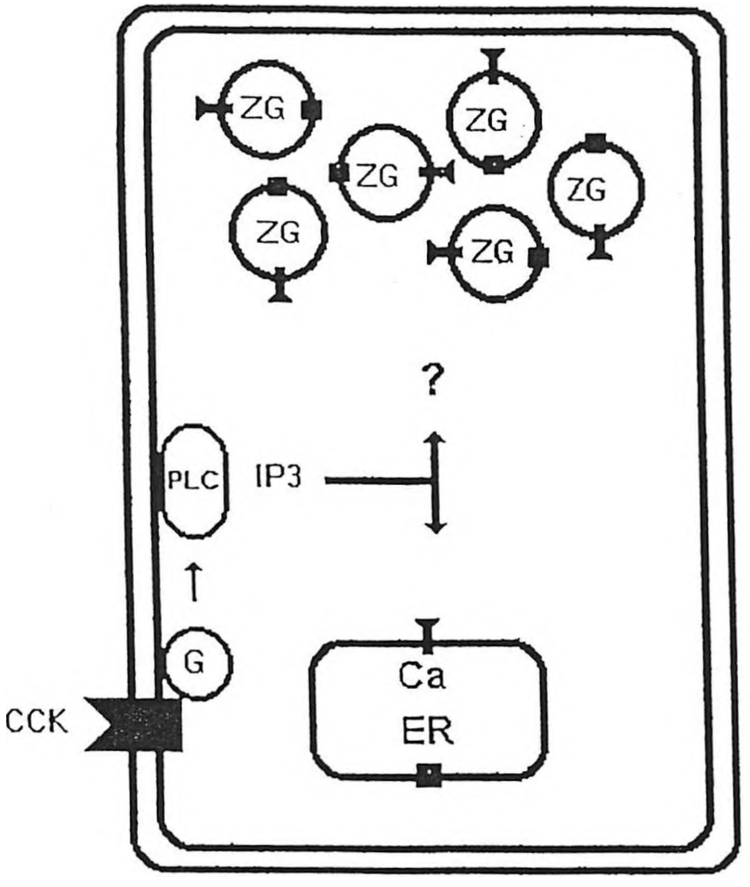
Mogami et al. (1997) have shown that Ca^{2+} can be depleted from apical intracellular stores and subsequently refilled by application of Ca^{2+} to the basal pole. This redistribution of Ca^{2+} between the basal and apical compartments suggest that the intracellular stores are continuous and not discreet subcompartments. Subramanian and Meyer (1997) have also shown, through the use of an ER directed fluorescent protein, that ER is one continuous compartment throughout the acinar cell. They found that the labeled protein has unrestricted access to all areas of the ER, providing further evidence for this continuity. A continuous ER, lacking subcompartments, should behave homogeneously as a Ca^{2+} store. However, other reports have suggested an asymmetric distribution of Ca^{2+} release receptors on the ER which could create physiological or functional Ca^{2+} subcompartments (Maranto, 1994). These calcium release receptors could include a variety of different IP_3 receptors or ryanodine receptors, all with different ligand binding affinities and responses.

Based on the uncertainties in the source of Ca^{2+} at the apical pole of pancreatic acinar cells during secretion, this project was designed to determine if

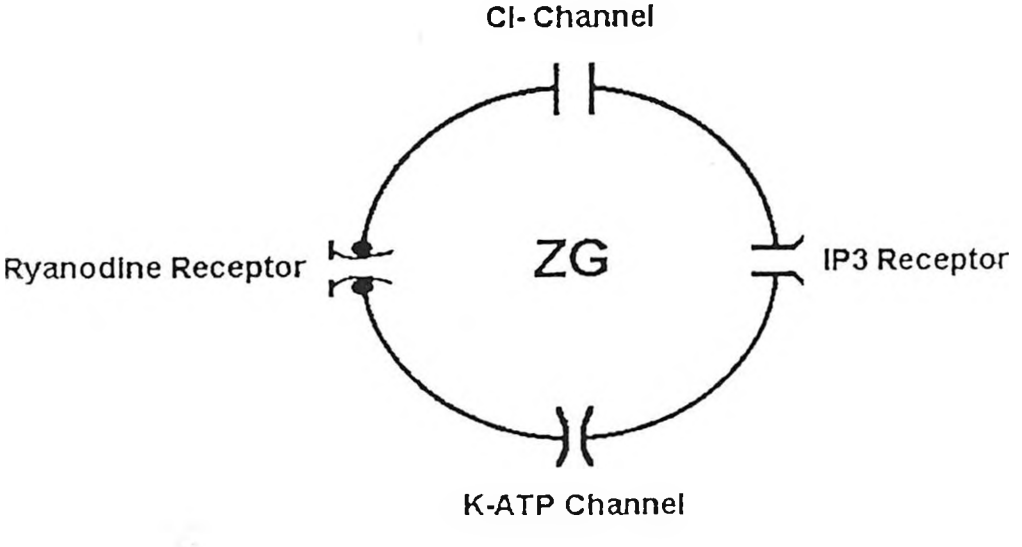
pancreatic secretory vesicles contain substantial amounts of Ca^{2+} and could then serve as an intracellular store for Ca^{2+}. Figure 1 illustrates a proposed physiological model for pancreatic Ca^{2+} regulation and the plausible transport pathways that may be relevant to this regulation. If vesicles do indeed possess high concentrations of Ca^{2+}, then the membranes of the vesicles must further possess the necessary Ca^{2+} release pathways to provide an avenue for Ca^{2+} efflux. The two potential release pathways that will be determined immunologically are the IP_3-sensitive Ca^{2+} channel and the ryanodine/cADP-ribose sensitive Ca^{2+} channel. In order to demonstrate the physiologically relevant function of the pathways for Ca^{2+} release, channel inhibitors will be utilized and the physiological behavior of the secretory vesicles monitored. The success of the inhibitors in an in vitro situation would provide evidence for Ca^{2+} flux occurs across the vesicle membrane. Furthermore, net ionic flux requires an electrically equivalent opposite flux of another ion. The candidate counterions for the Ca^{2+} efflux across the pancreatic secretory vesicle membrane include Cl^- (efflux) or K^+ (influx). As previously stated, both Cl^- and K^+ channels have been shown to exist at the vesicle membrane and experiments were designed to measure their contribution to Ca^{2+} efflux, as either could conceivably maintain the required electroneutrality and serve as a regulator of Ca^{2+} release.

Figure 1. This model illustrates the possible pathways by which Ca^{2+} stores may be liberated including the inositol-1,4,5-triphosphate (IP_3) receptor and ryanodine receptor. The model also illustrates possible counterion flux pathways that are known to reside within the secretory vesicle membrane and include a Cl^- channel and a K_{ATP} channel. It is possible that either import of K^+ or export of Cl^- are necessary components of Ca^{2+} efflux across the secretory vesicle membrane and may serve to regulate Ca^{2+} release and exocytosis.

Pancreatic Acinar Cell
Calcium Signaling



Ion Transport Pathways
In Zymogen Granule Membranes



MATERIAL AND METHODS

Secretory vesicles and plasma membranes were isolated from the pancreas of male Sprague-Dawley rats (125-175g) using previously described methods (DeLisle et al., 1984, Gasser and Holda, 1993). Briefly, the rats were killed by cervical dislocation while under ether anesthesia and the pancreas quickly removed and placed in ice-cold homogenization buffer of the following composition of 150 mM KCl solution, 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), titrated to pH 7.0 with KOH, 0.2 mM EGTA, and 0.2 mM $MgSO_4$. The pancreas was briefly minced and the tissue was suspended in homogenization buffer and disrupted with 5 strokes of a loose-fitting glass-Teflon homogenizer. The cells were subsequently lysed by nitrogen cavitation (750psi). This procedure disrupted virtually all the cells but left the secretory vesicles and other intracellular organelles intact. The homogenate was then supplemented with Percoll (45%v/v) while maintaining the original buffer concentrations and osmotic pressure. The secretory vesicles were isolated on the Percoll density gradient formed by centrifugation at 20,000x g for 20 minutes using a Sorvall SV-288 vertical rotor. The secretory vesicles formed a distinct high-density band at the bottom of the gradient.

Isolation of plasma membrane fractions

Plasma membranes were collected from the low density fraction of the Percoll gradient. A separation of the basolateral and apical membranes is made possible in part due to the tendency of nitrogen cavitation to lyse epithelial cells at the tight

junctions. This junctional matrix resides at the interface between the apical and basolateral domains of the plasma membrane. When secretory vesicle and plasma membranes were isolated from the same tissue, approximately 25% of the low density fraction was removed and diluted in a 2:1 ratio with resuspension buffer (300mM sucrose, 5 mM MOPS (titrated to pH 7.0 with NaOH), 0.1 mM EGTA and 0.1 mM $MgSO_4$). The diluted membrane fraction was then centrifuged at 33,000 x g for 30 minutes which yielded two distinct bands. The top band was removed, homogenized, and further diluted with 1:10 resuspension buffer followed by the addition of 30 mM $MgCl_2$. The suspension was placed on ice and stirred for 20 minutes allowing the Mg^{2+} to bind the intrinsic negative charges on the apical membrane segments and form large complexes due to divalent cation cross-linkages. The suspension was centrifuged at 2,500 x g for 25 minutes, pelleting higher density cell debris. The supernatant was recentrifuged at 20,000 x g for 20 minutes which pelleted the cross-linked apical membrane fragments while the basolateral membrane remained in the supernatant. The apical membrane pellets were resuspended in 0.5 ml resuspension buffer.

Evaluation of Membrane Fusion

Two methods were employed to evaluate fusion of secretory vesicles with target membranes. The first utilized light scatter techniques which measure the rate of dissolution of the condensed secretory vesicle matrix that accompanies fusion with a target membrane. Intact vesicles account for at least 90-95% of the optical density (OD) at 540 nm. The change in light scatter has been correlated with secretory vesicle concentration thereby providing a valid measurement of vesicle lysis (Gasser et al.,

1988a). Fusion is thought to alter the intravesicular environment that is necessary to maintain the macromolecular products in a condensed state. The subsequent solubilization of the secretory vesicle contents can be measured as the decrease in OD at 540nm. Experiments were performed by adding target membrane (15ug protein/ml) to cuvettes containing 3.0 ml of a solution containing either 300 mM sucrose or 150 mM KCl at 37°C. Purified secretory vesicles (25 ug protein/ml) were added to the suspension and the change in OD_{540} monitored with a Beckman DU-64 spectrophotometer equipped with a constant temperature chamber, an automatic 6 unit sampler, a computerized data capture system, and software for kinetic analysis. The time dependent change in OD_{540} associated with vesicle/apical membrane fusion was monitored for 15 minutes and always compared to the OD change associated with secretory vesicles alone as a baseline control. Half-times for fusion were calculated signifying the time required for a 50% decrease in OD and are reported as a rate constant representing the reciprocal of the half-times in hours. Data are reported as means $\pm$ SE of the mean.

The second method of monitoring membrane fusion employed the technique of florescent dequenching of the membrane probe octadecyl rhodamine B chloride (R18) (Hoekstra et al., 1984). R18 was obtained from Molecular Probes Inc. and a 10 mM stock solution was made with 100% ethanol. Purified secretory vesicles were incubated with 66 um R18 for 45 minutes at room temperature, giving a final probe concentration in the membrane of 6-9 mole%. Free R18 was subsequently removed by centrifugation of the labeled vesicles in a Percoll density gradient. Experiments were performed by

adding labeled vesicles (25 ug protein/ml) to a flourimetric cuvette containing either 300 mM sucrose or 150 mM KCl, 20 mM HEPES, titrated to pH 7.0 (with either KOH or NaOH), 0.2 mM EGTA, and 0.2 mM $MgSO_4$ at 37°C. The probe was excited at 560 nm, and the florescent signal was recorded at an emission wavelength of 590 nm using a Perkin-Elmer LS-3B spectrofluorimeter. After measurement of the fluorescent baseline, unlabeled target membrane (15 ug protein/ml) was added to the cuvette and the florescent signal followed for an additional 8 minutes. Fusion of the labeled vesicles with unlabeled target membranes results in lipid dilution and a relief in R18 self quenching with a subsequent increase in florescent intensity. Maximal fluorescence was determined by the addition of Triton X-100 to a final concentration of 0.2%. Simultaneous control experiments were run to correct for changes in secretory vesicle promoted light scatter.

Protein and Enzyme Assays

Protein was assayed using the Bio-Rad dye-binding assay based on the method of Bradford (1976). Mitochondrial contamination of membrane samples was measured as cytochrome c oxidase activity and was assayed according to the method described by Scarpa and Graziotti (1973). Endoplasmic reticulum contamination was determined by the glucose-6-phosphatase and NADPH cytochrome c reductase assay method of Beaufay et al. (1974) and Sottocasa et al. (1967).

Apical membrane content was quantified by measuring alkaline phosphatase activity (Gasser and Kirsschner, 1987). Membranes were added to a solution of 0.5 mM paranitrophenyl phosphate, 75 mM (2-[N-cyclohexylamino]-ethane sulfonic acid

(CHES) (pH 10.0)), and 10 mM $MgCl_2$ to initiate the reaction. 1 ml of 3.0 N NaOH was used to terminate the reaction. Absorbance was measured at 410 nm and the activity was calculated using an extinction coefficient of 18700 $M^{-1}cm^{-1}$. Basolateral membrane fragments were identified by measurements for Na-K-ATPase activity. The enzyme was quantified in a 0.5 ml mixture containing 100 mM NaCl, 20 mM KCl, 6 mM $MgSO_4$, 50 mM imidazole (pH 7.2), and 4 mM Na_2-ATP. Samples were incubated for 30 minutes at 30°C and the reaction was then terminated by placing the tubes into an ice bath and adding 1.0 ml of 1.25% acid molybdate. The difference between PO_4^{2-} release in tubes with and without 1.0 mM ouabain represented the NaK-ATPase activity. A modification of the Fiske-SubbaRow method was used to quantify inorganic phosphate (Peterson, 1978).

Evaluation of Vesicular Ionic Permeability

The ionic permeability of isolated secretory vesicles was determined indirectly by the method of ionophore-dependent granule lysis in a defined salt solution (Gasser and Hopfer, 1990; Gasser and Holda, 1993). Creation of a cation or anion permeability through the use of ionophores initiates a driving force for transport of the counterion through an endogenous membrane channel. The technique measures permeability based on solute driven osmotic influx of fluid and the kinetics of the subsequent swelling and lysis of the vesicle as measured by the decrease in OD_{540}. Experiments were conducted with a Beckman DU-64 spectrophotometer equipped with a constant temperature chamber, 6-unit sample changer, computerized data capture system, and software for kinetic analysis. Added ionophores were taken from stock solutions in

100% ethanol; however, ethanol concentrations in the vesicle suspension never exceeded 1%.

To measure anion permeability, the secretory vesicles were suspended in a 37°C solution consisting of 150 mM KCl, 20 mM HEPES (titrated to pH 7.0 with KOH), 0.2 mM EGTA, and 0.2 mM $MgSO_4$. A cation permeability was created by the addition of valinomycin (K^+ ionophore) at a concentration of 10μg/ml. The endogenous Cl^- transport becomes the rate limiting step under these conditions as electroneutrality must be maintained for net solute transport. Cl^- specific transport was verified using the Cl^- channel blocker 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS). The cation permeability of isolated secretory vesicle was measured as described in detail previously (Gasser et al., 1988). This technique employed permeabilizing the membrane with ionophores specific for creating a Cl^- conductance. The vesicles were suspended in a 37°C salt solution of 150 mM KCl, 20 mM HEPES (titrated to pH 7.0 with KOH), 0.2 mM EGTA, and 0.2 mM $MgSO_4$. The Cl^- permeability was increased with the use of the chloride-hydroxyl exchanger tripropyltin (TPT; 10 ug/ml) and a protonophore 3, 3', 4,'5-tetrachloro-salicylanide (TCS; 10ug/ml). The combined ionophores create a chloride conductance thereby imposing a driving force for cation flux. Under these conditions, the relative contribution of various cations to the rate of vesicle swelling was determined by selectively blocking the endogenous Ca^{2+} channels (ruthenium red, $LaCl_3$, heparin, or $NiCl_2$) and K^+ channels (ATP, glybenclamide). Net salt flux alters the intravesicular environment causing protein solubilization, osmotic water influx, and vesicle lysis at a rate limited by the endogenous Ca^{2+} or K^+ flux.

Measurement of Vesicle Ca^{2+} Concentration

The Ca^{2+} concentration of secretory vesicles was measured using the Ca^{2+}-sensitive fluorescent probe FURA-2 (Molecular Probes Inc.). A dual excitation ratiometric method was used to measure the Ca^{2+}-dependent change in FURA-2 fluorescence. FURA-2 was excited at both the Ca^{2+}-insensitive or isobestic wavelength of 360 nm and at the Ca^{2+}-sensitive wavelength of 380 nm. The emitted fluorescence was measured at 510 nm. Ca^{2+} concentration was calculated based on the ratio of these two readings. To determine intravesicular Ca^{2+} concentration, secretory vesicles were suspended in a solution containing 10 μM FURA-2, vesicles were lysed with 0.01% Triton X-100, and the ensuing change in solution fluorescence recorded. Intravesicular Ca^{2+} concentration was back-calculated based on the amount of vesicle protein added to the suspension and the known amount of protein per vesicle to give an estimate of the number of vesicles. Finally, using the known intravesicular volume per vesicle, the total starting volume could be calculated to estimate the intravesicular Ca^{2+} concentration that would be required to account for the recorded Ca^{2+} following dilution (upon vesicle lysis) into the 3.0 ml suspension solution.

Identification of IP_3 and Ryanodine Receptor Ca^{2+} release pathways

Hydrophobic membrane proteins, including both IP_3 and ryanodine receptor Ca^{2+} channels, were isolated from secretory vesicle preparations by incubation with Triton X-114 (Bordier, 1981). This method relies on the ability of the detergent to separate hydrophobic proteins (associated with the membrane) from hydrophilic proteins via cloud point separation. The Triton X-114 extracted vesicle protein were prepared for

immunoblot analysis by dilution in Laemmli reducing buffer (0.08 M Tris-HCl, pH 6.8, 10% glycerol, 0.01% bromophenol blue, 2% SDS, and 5% β-mercaptoethanol) (Laemmli, 1970). The sample was loaded onto a discontinuous SDS-polyacrylamide gel (3.0% stacking and 5.0% separating) and electrophoresis performed at 100 volts for 60 minutes. Proteins were subsequently electroblotted onto a pure nitrocellulose membrane (0.2 μm pore size) for 1 hour at 100 volts at 4C. After electroblotting, the nitrocellulose sheet was blocked in 5% non-fat dry milk in 0.05% Tween-20 and TBS (500 mM NaCl, 20 mM Tris, pH 7.5) for 1 hour at room temperature or overnight at 4°C.

Western blots were performed using a rabbit polyclonal anti-rat IP_3 receptor antibody and an anti-rat ryanodine receptor antibody obtained from Upstate Biotechnology Inc. Following blocking, nitrocellulose blots were incubated in a solution containing a 1:1000 dilution of this antibody in 1% non-fat dry milk, 0.05% Tween-20, and TBS for 1 hour at room temperature. The blots were then washed 3 times for 10 minutes each in 0.05% Tween-20 and TBS at room temperature. Primary antibody binding to the secretory vesicle IP_3 and ryanodine receptors were detected using a 1:2000 dilution of stock horseradish peroxidase-labeled donkey anti-rabbit IgG (Amersham Co., Inc). The secondary antibody was incubated in 1% non-fat dry milk in 0.05% tween-20 and TBS with the blot for 1 hour at room temperature. Following the incubation with secondary antibody, the blots were washed 4 times for 10 minutes each in 0.05% Tween-TBS. Following the final wash, the blot was immediately incubated in luminol developer solution and the specific proteins detected by chemiluminescence and recorded using blue light-sensitive autoradiographic ECL Hyperfilm.

RESULTS

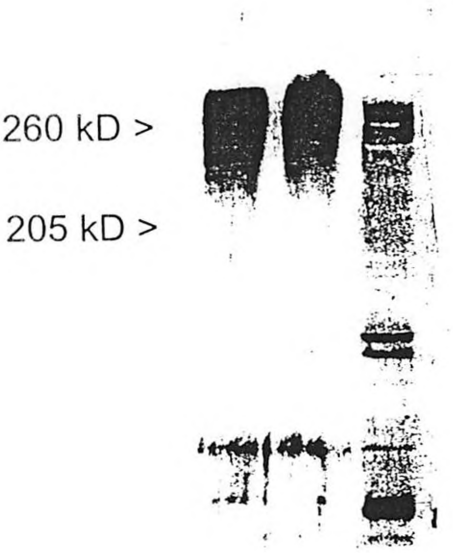
Secretory vesicles isolated from the rat pancreas were enriched approximately 5.4-fold and were stable when suspended in solution at 37°C. To determine the intravesicular Ca^{2+} concentration, 0.82 mg secretory vesicle protein was added to a cuvette containing 10μM FURA-2. The vesicles were lysed with Triton X-100 releasing the granule contents (including Ca^{2+}) into a 3ml solution. Ratiometric analysis showed an increase in the FURA-2 ratio of approximately 12% equivalent to 26nM Ca^{2+} in the cuvette. Intravesicular Ca^{2+} was subsequently calculated based on 0.31 pg protein per vesicle and the average intravesicular volume of 0.7 fL. Based on these assumptions and FURA-2 values, intravesicular Ca^{2+} was estimated to be 25mM. This is a significant amount of Ca^{2+}, representing a gradient that is 10,000 times higher in secretory vesicle lumen versus the cytoplasm. Since this level of Ca^{2+} is sufficient to act as an intracellular store, further experiments were designed to determine if the relevant Ca^{2+} release pathways exist in the secretory vesicle membrane.

Isolated secretory vesicles were solubilized in 0.1%SDS, centrifuged at 15,000 x g for 15 minutes, and the supernatant diluted 1:3 with Laemmli reducing electrophoresis buffer. 20μg of vesicle protein was separated by SDS-PAGE and blotted to nitrocellulose. Immunoblotting with either antibodies to the IP_3 or the ryanodine receptor resulted in the appearance of protein bands at 260 kD and 550 kD, respectively (figure 2). Positive controls obtained from ovine cerebellum

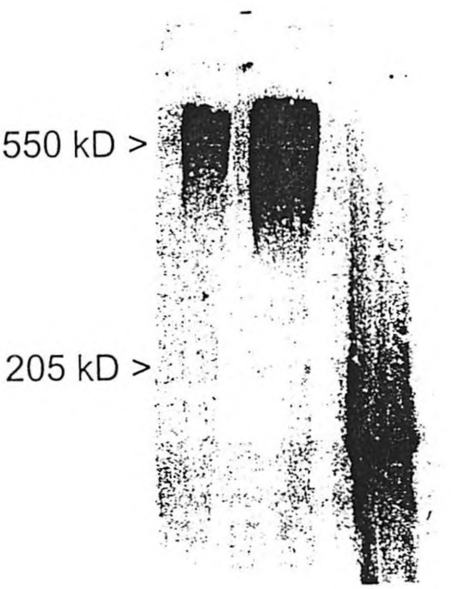
Figure 2. This is a Western blot of proteins extracted from highly purified membranes of rat pancreatic secretory vesicles. The proteins were separated by SDS-PAGE, electroblotted onto nitrocellulose, and subsequently probed with antibodies specific for either IP_3 (**left**) and ryanodine receptor (**right**). The antibodies were visualized by enhanced chemiluminescence.

Western Blots of Zymogen Granule Membrane Proteins

IP_3 Receptor



Ryanodine Receptor



microsomes and skeletal muscle sarcoplasmic reticulum confirmed the location of the Ca^{2+} release pathways. Therefore, secretory vesicles contain substantial amounts of Ca^{2+} as well as the appropriate pathways to deliver that Ca^{2+} to the cytoplasm.

To determine if the Ca^{2+} release pathways identified by western blotting were functional, the ionic permeability of the vesicle membrane was analyzed. A cation flux was initiated by the addition of the ionophores TPT/TCS. These ionophores create a Cl^- conductance and conditions whereby vesicle swelling and lysis is rate limited by the endogenous cation conductance. To determine the specific cation involved, secretory vesicles were treated with blockers specific to either the Ca^{2+} release pathways or the K_{ATP} channel. By this approach, a decrease in the relevant cation permeability will be evident by a corresponding decrease in the rate of vesicle swelling and lysis.

Ruthenium red specifically inhibits the ryanodine receptor and caused a decrease in the rate of cation dependent vesicle lysis (figure 3). Consistent with the CICR mechanism of ryanodine receptors, mM concentrations of Ca^{2+} also reduced the rate (table 1). This result suggests that the ionophore induced granule lysis was dependent on a Ca^{2+} flux. Furthermore, nonspecific Ca^{2+} channel blockers such as $NiCl_2$ and $LaCl_3$ also inhibited or reduced the cation dependent vesicle swelling and lysis consistent with a substantial Ca^{2+} permeability (table 1; figure 3). Since Ca^{2+} channel blockers prevented swelling and lysis, the results then imply that Ca^{2+} efflux occurs in conjunction with the imposed Cl^- flux. Alternatively, when a K^+ permeability was artificially imposed

Figure 3. This graph illustrates the effect of $LaCl_3$ (330μM) and ruthenium red (22.0μM) on Ca^{2+} dependent vesicle lysis in KCl suspensions. Vesicle lysis was measured as the decrease in optical density (OD_{540}) after the addition of tripropyl-tin (TPT) and tetrachlorosalicylanilide (TCS) to promote a Cl^- conductance across the vesicle membrane. Secretory vesicles were pretreated with either $LaCl_3$ or ruthenium red prior to the addition of TPT and TCS. Secretory vesicles were suspended in a solution consisting of 150 mM KCl, 20 mM HEPES (pH 7.0), 0.2 mM EGTA, and 0.2 mM $MgSO_4$. The figure shows the results of a single experiment and is representative of 9 other similar experiments.

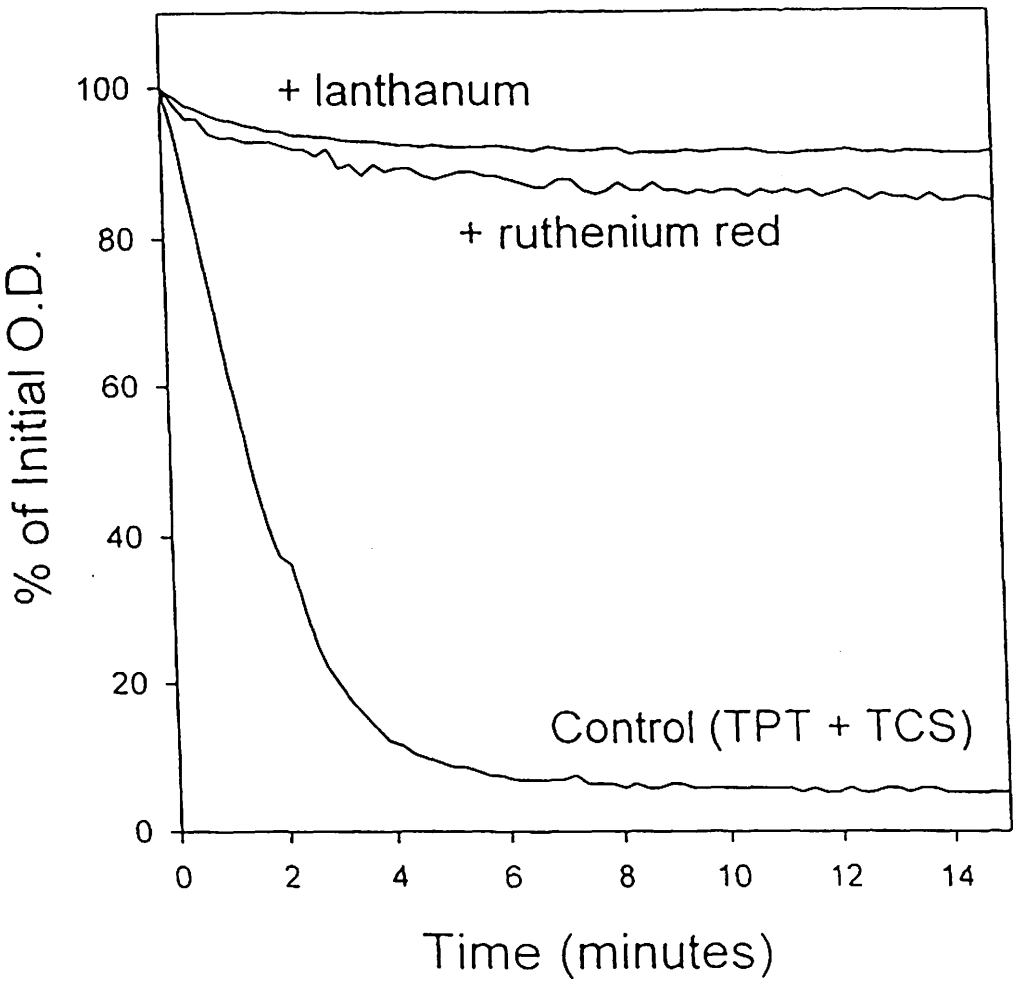

+ lanthanum
+ ruthenium red
Control (TPT + TCS)
% of Initial O.D.
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (minutes)

Table 1

Effect of Ca^{2+} release pathway inhibitors on secretory vesicle cation dependent swelling and lysis in vitro.

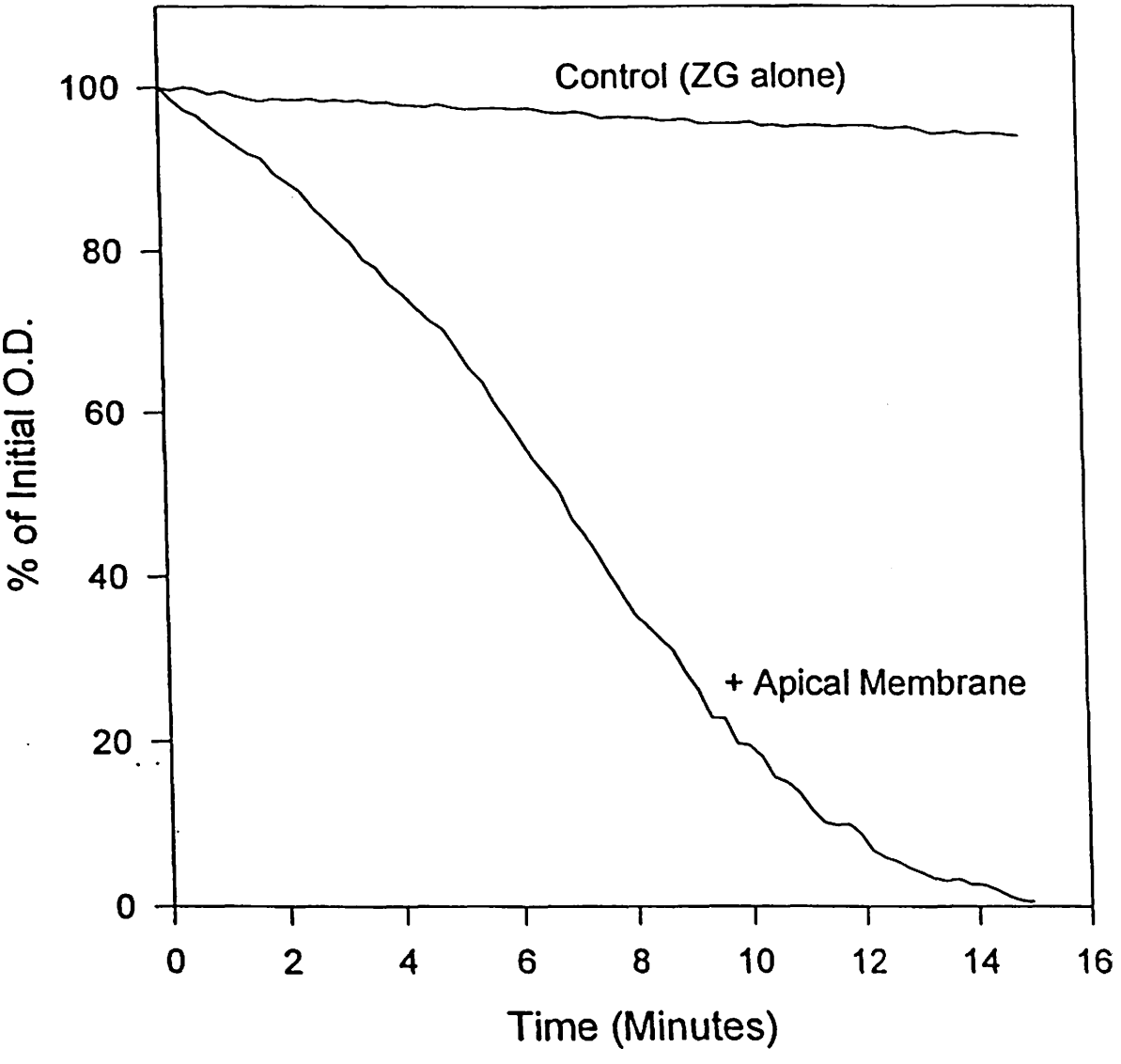
Reagent	Concentration	% Inhibition
$LaCl_3$	0.4 mM	50%
Ruthenium red	0.022 mM	50%
$NiCl_2$	6.0 mM	50%
Heparin	67 units/ml	21%

Secretory vesicle cation dependent swelling and lysis was measured as the decrease in OD_{540} after the addition of TPT/TCS to a suspension of secretory vesicles. The selected reagents are known Ca^{2+} channel blockers and the results reflect the Ca^{2+} contribution to cation dependent swelling and lysis. Inhibition is based on the difference of the rate in decrease of OD_{540} between a control experiment without the reagent and an identical matched experiment containing the reagent. This table represents results of least 3 experiments with each reagent.

with the ionophore valinomycin, there was no increase in the rate of swelling and lysis over the controls. Furthermore, inhibition of the endogenous K^+ channel with ATP or glybenclamide did not decrease the rate of swelling and lysis over the controls. These results suggest that the Ca^{2+} efflux across the vesicle membrane dominates under these conditions, with little contribution by the K^+ channel.

Release of Ca^{2+} from the secretory vesicle may also imply a role for Ca^{2+} in exocytotic membrane fusion. Fusion between isolated secretory vesicles and apical plasma membrane was measured in vitro by two techniques. Isolated secretory vesicles are very stable when suspended in either a KCl or sucrose solution at 37°C, exhibiting half-lives of 75.6$\pm$ 7.4 minutes and 87.2$\pm$ 6.1 minutes respectively. This low intrinsic rate of vesicle lysis allowed membrane fusion to be measured by the change in OD_{540} after the addition of target membrane. The target membranes consisted of pancreatic apical membrane, liver plasma membrane, and other secretory vesicles. Fusion of the secretory vesicle with a target membrane is thought to alter the environment of the vesicle lumen causing a dissolution of the condensed protein matrix which can be measured by a decrease in OD or an increase in light scatter. Figure 4 illustrates the stability of secretory vesicles (30 μg/ml protein) and the subsequent rate of membrane fusion following addition of pancreatic apical membrane (15μg/ml protein). The mean change in the rate of vesicle matrix dissolution associated with fusion went from 75.6 minutes in the control without the target membrane to 8.1 minutes with the target membrane.

Figure 4. This graph represents fusion of secretory vesicles with apical plasma membrane. Membrane fusion was measured as the decrease in optical density (OD_{540}) after the addition of apical membranes (15μg protein/ml) to a suspension of secretory vesicles (25μg protein/ml). The control represents the endogenous decrease in OD_{540} of secretory vesicles in the absence of apical membrane. Secretory vesicles and apical membranes were suspended in a 37°C solution consisting of 150mM KCl, 20mM HEPES (pH 7.0), 0.2mM EGTA, and 0.2mM $MgSO_4$.


Control (ZG alone)
+ Apical Membrane
% of Initial O.D.
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Time (Minutes)

The second technique used to measure membrane fusion was based on the rate of R18 dequenching and lipid mixing between the fusing membranes. Dilution of the R18 fluorescent probe upon fusion can be measured as an increase in fluorescent intensity. Figure 5 illustrates the change in R18 fluorescence when labeled secretory vesicles are mixed with unlabeled apical plasma membrane, liver plasma membrane, or other secretory vesicles. The results show a dramatic increase in fluorescent intensity when unlabeled apical membrane is added, but little if any change when liver membrane or unlabeled secretory vesicles are added. This implies that secretory vesicles will only specifically fuse with pancreatic apical membrane and that nonspecific transfer of R18 from labeled to unlabeled membranes does not occur. Together these techniques (change in OD_{540} and R18 dequenching) provide overlapping evidence for secretory vesicle-apical membrane fusion in vitro.

To determine if Ca^{2+} has a role in this process, fusion experiments were performed in the presence and absence of Ca^{2+} channel blockers. Figure 6 illustrates the effect of ruthenium red and $LaCl_3$ on the rate of secretory vesicle and apical membrane fusion. The results show that both agents prevent fusion, suggesting Ca^{2+} efflux is a prerequisite for exocytosis. Concentrations of inhibitors required to decrease the rate of fusion by 50% are listed in table 2. In general, these K_i values are similar to the concentrations reported in the literature for blocking Ca^{2+} release pathways (Coronado et al., 1994; Tsunoda, 1993). Heparin is a specific blocker of the IP_3 sensitive Ca^{2+} release pathway. The maximum inhibition of in vitro membrane fusion in the presence of heparin

Figure 5. This graph illustrates the ability of secretory vesicles or liver plasma membrane to serve as a fusion target as measured by R18 lipid mixing. Membrane fusion was measured as the percent of fluorescence dequenching accompanying R18 probe dilution/lipid mixing after the addition of unlabeled membranes (15μg protein/ml) to a suspension of R18 labeled secretory vesicles (25μg protein/ml). After monitoring baseline fluorescence, unlabeled membranes (liver plasma membranes or unlabeled secretory vesicles) were added and the increase in fluorescence was followed for an additional 8 minutes. Total fluorescence is the value obtained after solubilization of the membranes were suspended in a solution consisting of 150mM KCl, 20mM HEPES (pH 7.0), 0.2mM EGTA, and 0.2mM $MgSO_4$.

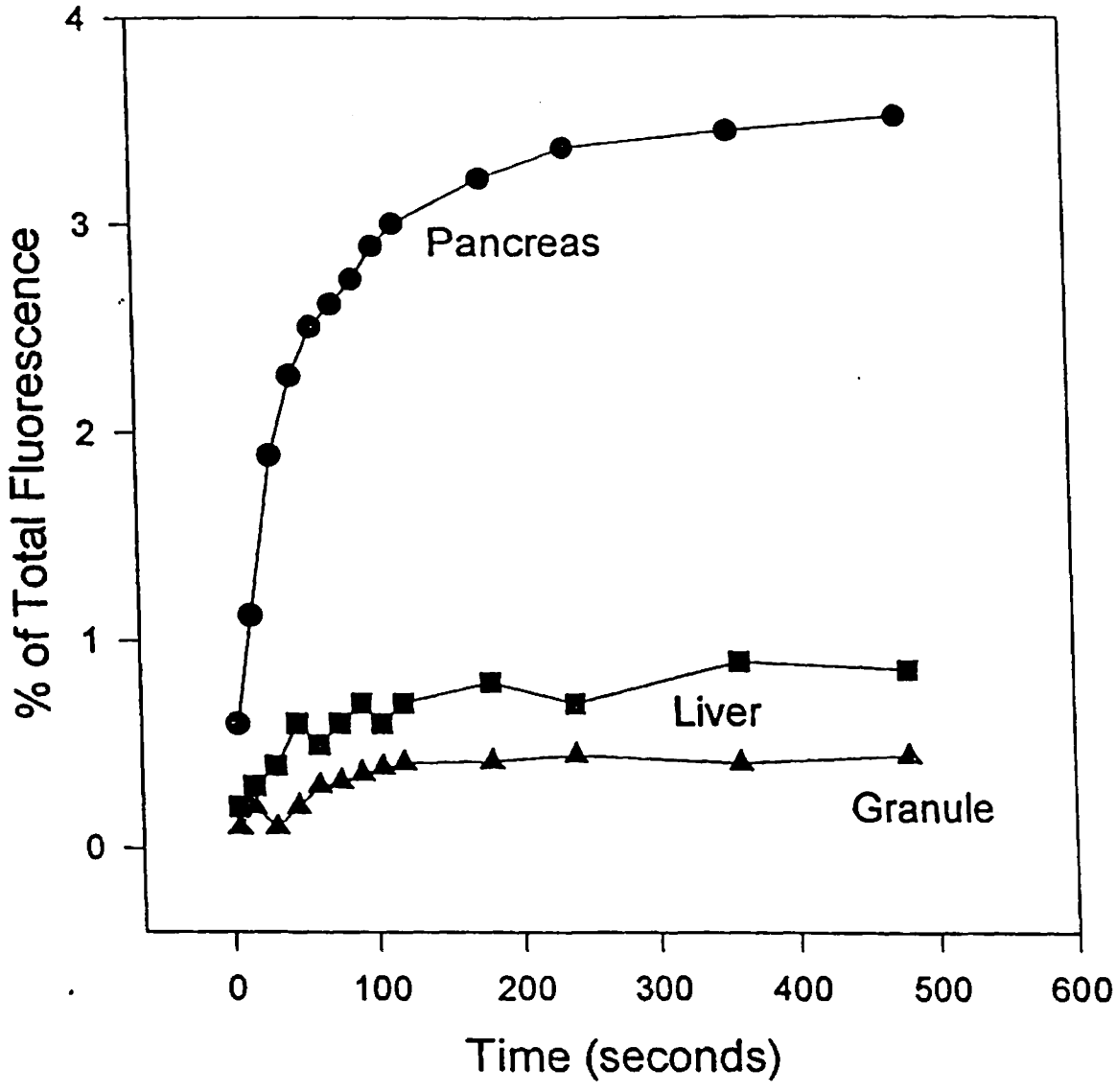

4
3
2
1
0
% of Total Fluorescence
Pancreas
Liver
Granule
0
100
200
300
400
500
600
Time (seconds)

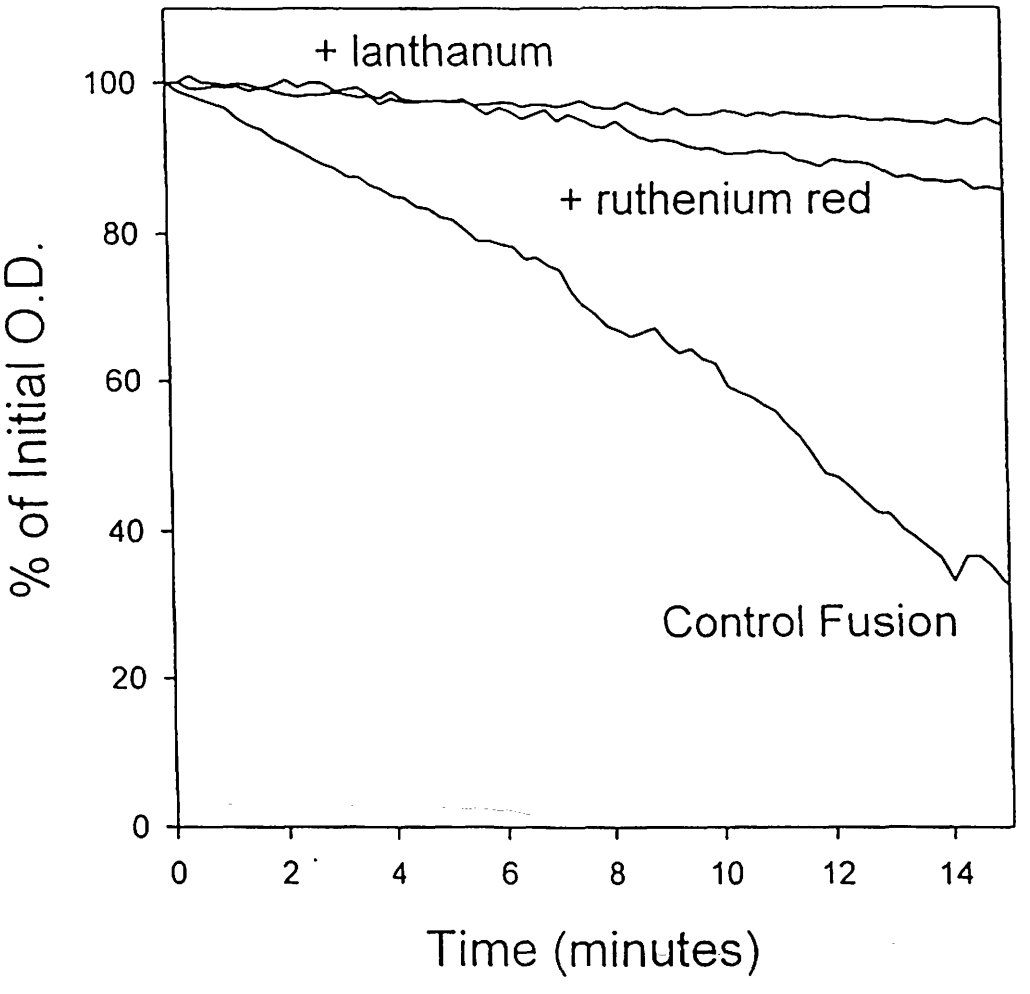
Table 2

Effect of Ca^{2+} release pathway inhibitors on secretory vesicle fusion in vitro.

Reagent	Concentration	% Inhibition
$LaCl_3$	0.150 mM	50%
Ruthenium red	0.025 mM	50%
$NiCl_2$	5.0 mM	50%
Ca^{2+}	5.0 mM	50%
Heparin	67 units/ml	43%

Membrane fusion was measured as the decrease in OD_{540} after the addition of apical membranes to a suspension of secretory vesicles. Inhibition is based on the difference of the rate in decrease of OD_{540} between a control experiment without the reagent and an identical matched experiment containing the reagent. This table represents results of least 3 experiments with each reagent.

Figure 6. This graph illustrates the effect of $LaCl_3$ (250μM) and ruthenium red (33.3μM) on secretory vesicle-apical membrane fusion in KCl suspensions. Membrane fusion was measured as the decrease in optical density (OD_{540}) after the addition of apical membranes (15μg protein/ml) to a suspension of secretory vesicles (25μg protein/ml). Secretory vesicles were pretreated with either $LaCl_3$ or ruthenium red prior to the addition of apical membranes. Secretory vesicles and apical membranes were suspended in a solution consisting of 150 mM KCl, 20 mM HEPES (pH 7.0), 0.2 mM EGTA, and 0.2 mM $MgSO_4$. The figure shows the results of a single experiment and is representative of 10 other similar experiments.

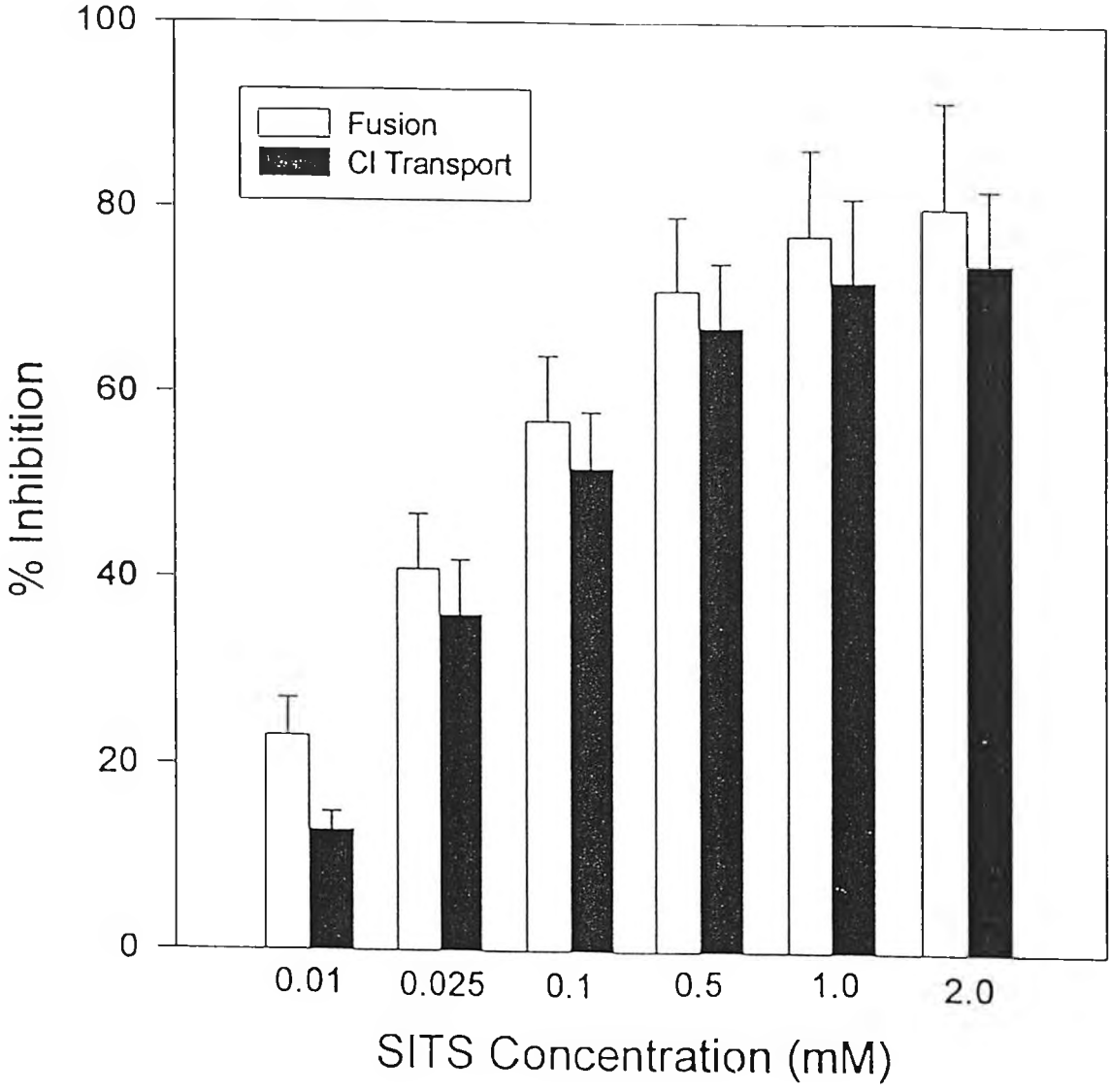

+ lanthanum
+ ruthenium red
Control Fusion
% of Initial O.D.
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (minutes)

was 43%. This suggests that the IP_3 Ca^{2+} release pathway contributes less to the total Ca^{2+} efflux than the ryanodine sensitive Ca^{2+} release pathway.

Since the data implied that Ca^{2+} efflux was necessary for membrane fusion, further experiments were designed to determine if the effect is a direct result of the increase in external Ca^{2+} or the decrease in intravesicular Ca^{2+}. Exogenously added Ca^{2+} (0.1-2.0 mM) did not increase fusion efficiency, nor did it reverse the $LaCl_3$ or ruthenium red inhibition of fusion (data not shown). This result implies that an increase in external Ca^{2+} is not a prerequisite for membrane fusion but rather a decrease in intravesicular Ca^{2+} is the required signal for fusion.

The above data suggests that Ca^{2+} efflux is required for exocytotic membrane fusion. Since a counterion is required for net Ca^{2+} efflux, experiments were designed to determined if Cl^- efflux or K^+ influx fulfilled this role. Blocking the Cl^- channel should inhibit membrane fusion in the same fashion that fusion was inhibited when the Ca^{2+} channel was blocked if Cl^- serves as a counterion. SITS, a blocker of vesicle Cl^- channels, caused a dose dependent decrease in both Cl^- permeability and fusion efficiency (figure 7) . ATP and glybenclamide, blockers of the K_{ATP} channel, did not cause a comparable decrease in the fusion rate. Therefore, the data suggest that Cl^- efflux and not K^+ influx across the secretory vesicle membrane serves as the counterion to Ca^{2+} efflux leading to exocytosis and membrane fusion.

Figure 7. This histogram illustrates 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS) inhibition of secretory vesicle-apical membrane fusion and Cl^- transport. The secretory vesicles were pretreated with the listed concentrations of SITS and percent inhibition was determined using rates for transport and fusion in the presence versus the absence of the inhibitor. Secretory vesicles were suspended in a solution consisting of 150 mM KCl, 20 mM HEPES (pH 7.0), 0.2 mM EGTA, and 0.2 mM $MgSO_4$.


100
80
60
40
20
0
% Inhibition
Fusion
Cl Transport
0.01
0.025
0.1
0.5
1.0
2.0
SITS Concentration (mM)

DISCUSSION

Calcium is a ubiquitous intracellular second messenger, controlling a vast variety of tissue specific physiological mechanisms. Due to its importance, the processes that regulate its intracellular concentration have been intensely studied (see review by Tsunoda, 1993). A breakthrough in understanding its control came when Streb et al. (1984) demonstrated that IP_3 could cause the release of Ca^{2+} from the ER (Streb et al., 1984). Since that discovery, it has become generally accepted that agonists evoke Ca^{2+} signaling by activating enzymes to promote IP_3 generation and the subsequent release of Ca^{2+} from intracellular stores in nonexcitable cells (Berridge, 1993).

The pancreas is comprised of highly polarized epithelial cells that also generate IP_3 in response to gastrointestinal hormones. Physiological levels of these hormones evoke localized increases of Ca^{2+} in the apical pole that may subsequently spread to the basolateral regions of the cell (Thorn et al, 1993; 1994). These localized apical increases in Ca^{2+} are not due to exclusive localization of hormone receptors near the apical pole. However, the increase in Ca^{2+} is a function of IP_3 and IP_3-sensitive stores as microinjection of IP_3 directly into acinar cells induces the same Ca^{2+} release patterns (Kassai, 1995). This result suggests that a highly responsive IP_3 sensitive store of Ca^{2+} exists in the apical pole of pancreatic acinar cells. Lee et al. (1997) have also shown that Ca^{2+} waves are initiated in the apical pole of pancreatic acinar cells, which subsequently propagate to the cell periphery and the basal pole. In that study, the cells were shown to express the three known IP_3 receptor subtypes and were

localized to regions adjacent to the plasma membrane. Numerous electron microscopic studies of pancreatic acinar cells have shown the apical pole to be densely packed with secretory vesicles and that the basal pole is dominated by the ER and nucleus (Kern, 1993; Gorelick and Jamieson, 1994). These observations lead to the hypothesis that secretory vesicles may serve as the source of apical Ca^{2+}.

Previous attempts to explore this hypothesis have been inconsistent due in part to differences in secretory vesicle isolation procedures, vesicle stability (which may be related to the luminal Ca^{2+} content), and Ca^{2+} monitoring techniques. Gerasimenko et al. (1996) have shown Ca^{2+} release from isolated secretory vesicles in response to both IP_3 and cADP-ribose. Ca^{2+} release was monitored by the change in Fluo-3 fluorescence. Ca^{2+} was not released from isolated secretory vesicles in response to the Ca^{2+} ATPase inhibitor thapsigargin. However, thapsigargin in vivo causes a preferential increase in Ca^{2+} at the basal pole suggesting an effect on the ER store in that region. Further evidence for secretory vesicles serving as a Ca^{2+} store in pancreatic acinar cells came from studies which demonstrated that an acidic environment is required for Ca^{2+} signaling. (Gonzalez et al., 1997). Secretory vesicles are the dominant acidic compartment in acinar cells with a luminal pH of approximately 6.3 (Gasser et al., 1988). Furthermore, Titievsky et al., (1996) showed that a decrease in vesicle acidity inhibited Ca^{2+} signaling in pancreatic acinar cells, once again suggesting that the vesicles may serve as a Ca^{2+} store and that the acidic pH is required to maintain that store.

Conversely, Yule et al. (1997) presented immunohistological evidence that IP_3 receptors were not located on secretory vesicle membranes and therefore could not serve as the site for Ca^{2+} release. However, as with the results of Gerasimenko (1996), the preparation did not respond to thapsigargin, once again suggesting that the Ca^{2+} release site is not the ER. Finally, Mogami et al. (1997) presented evidence showing that the Ca^{2+} store in the pancreas was continuous between the basal and apical pole. Their results would imply that the apical release of Ca^{2+} is due to the localization of a portion of the ER to that region of the cell.

Results of the present study, using highly purified and enriched secretory vesicles, show evidence in support of Ca^{2+} release pathways being expressed on secretory vesicles. Western blots of membrane protein yielded proteins with molecular weights and antibody reactivity consistent with IP_3 and ryanodine receptors. Conclusions cannot be drawn about the specific receptor subtypes found on the secretory vesicles, since the antibodies were only specific for the conserved concensus region of the protein. It is not likely that the results were due to contamination of the vesicle preparations with ER or nuclear membrane based on enzymatic and microscopic analyses of the vesicle samples.

Three subtypes of the IP_3 receptors have been shown to be expressed in various tissues (Mikoshiba, 1997). The pancreas has been shown to contain all 3 of these subtypes (Shuttleworth, 1997). Nathanson et al. (1994) suggested the type 3 form may preferentially exist at the apical pole of the pancreatic acinar

cell, whereas the types 1 and 2 only exist at the basal pole. The histochemical results imply that the type-3 receptor associates with secretory vesicles, and the types-1 and 2 associate with the ER and nucleus. These observations of differential receptor localization may explain in part the localized pattern of Ca^{2+} release and the subsequent spreading of the response to adjacent regions of the cell, as different receptors subtypes will preferentially respond to the different IP_3 levels and cytosolic Ca^{2+} concentrations.

Based on the differences in IP_3 receptor subtypes and their localization within pancreas acinar cells, the present study's demonstration of ryanodine receptors in the vesicle membrane was unexpected. IP_3 has previously been shown by several studies to initiate the physiological pattern of Ca^{2+} release (Thorn et al., 1993). However, control of the ryanodine receptor may be mediated by IP_3-sensitive Ca^{2+} release followed by a CICR response from the vesicle ryanodine receptors. These results are consistent with those of Gerasimenko et al. (1996) which showed a cADP-ribose mediated release of Ca^{2+} from secretory vesicles, suggesting the involvement of a ryanodine receptor. Thorn et al. (1994) also showed that cADP-ribose regulated ryanodine receptors which were involved in promoting cytosolic Ca^{2+} concentration oscillations in the pancreatic acinar cells. However, the specific subcellular location of the ryanodine receptor was unidentified in that study. Studies of parotid acinar cells have shown a ryanodine sensitive Ca^{2+} release from microsomal vesicles and that this release could be further promoted by cADP-ribose (Ozawa and Nishiyama, 1997).

Cyclic ADP-ribose is a recently discovered second messenger derived from NAD^+ (Kim et al., 1993). Most nonexcitable cells are thought to synthesize and release cADP-ribose as a means to increase the open probability of the ryanodine receptor Ca^{2+} channel (Galione et al., 1991). It is the type 3 ryanodine receptor that responds to physiological levels of cADP-ribose. Furthermore, it has been shown that the type 3 receptor is found preferentially in nonexcitable cells, such as the pancreas (Coronado et al., 1994). Due to the pharmacological response of the secretory vesicles (inhibition of cation dependent lysis and membrane fusion) to ruthenium red, a specific inhibitor of the ryanodine receptor (Meissner, 1984), the present physiological results are also consistent with ryanodine receptor control of Ca^{2+} release in pancreatic acinar cells.

The data also show that the addition of exogenous Ca^{2+} does not promote an increase in fusion efficiency. Rather external Ca^{2+} was shown to decrease the fusion rate through what may have been a negative feedback on the open probability on the ryanodine receptor. These results suggest that the role of Ca^{2+} efflux to the fusion event may not be directly related to an increase in cytoplasmic Ca^{2+}, but that efflux may solely serve to decrease the intravesicular Ca^{2+} concentration. Since condensation of the vesicle protein matrix is Ca^{2+}-dependent (Fernandez et al., 1991), a decrease in luminal Ca^{2+} concentration may induce the decondensation of this protein core. Subsequent osmotic influx associated with protein solubilization may in turn induce vesicle swelling, an action previously shown to increase exocytotic fusion efficiency (Moser et al., 1995). However, in vivo studies have clearly shown that decreases in

cytoplasmic Ca^{2+} cause a decrease in secretion and exocytosis. These conflicting results may be resolved when the differences between the in vivo and in vitro environment are considered. In vitro, the driving force for fusion may be the requisite vesicle swelling promoted by the Ca^{2+} efflux. In vivo, this driving force may remain a requirement; however, the increase in cytoplasmic Ca^{2+} associated with vesicle efflux may also be required to activate associated or accessory mechanisms including trafficking of vesicles to the plasma membrane (Shuttleworth, 1997) and lipid metabolic pathways responsible for changes in membrane fluidity (Gasser and Holda, 1994).

In addition to the demonstrated Ca^{2+} channels, secretory vesicles are known to possess many other varieties of ion channels including ClC-2 Cl^- channels (Gasser and Hopfer, 1990), K_{ATP} channels (Gasser and Holda, 1993), Na^+/H^+ exchangers (Anderie and Thevenod, 1996), and Cl^-/HCO_3^- exchangers (Gasser et al, 1988). The physiological role of these transport pathways have not been clearly defined; although past evidence suggested a role in fluid production and secretion. Several studies have presented evidence that channel dependent vesicle swelling increases the efficiency of fusion (Moser et al., 1995; Zimmerberg et al., 1988). Net Ca^{2+} transport by the secretory vesicle may promote vesicle swelling but would require a counterion to maintain the electroneutrality of the vesicle membrane. The model could accommodate either Cl^- efflux or K^+ influx as necessary counterions to Ca^{2+} efflux from the vesicle. The present results would suggest that the Cl^- channel but not the K^+ channel performs this function.

The ER is a widely studied Ca^{2+} store and consistent with the current results, Ca^{2+} uptake and release was also shown to rely on Cl^- as a counterion (Kourie, 1997). ER Cl^- channels have unique characteristics including modulation of the open channel probability by ATP and its analogs, Ca^{2+} concentration, and inositol phosphates (Kourie et al., 1997; Kourie, 1997). The characteristics of the pancreatic secretory vesicle's Cl^- channel differ in their pharmacological behavior, namely in its responsiveness to inositol phosphates, although their physiological role may be the same. The ER concentrates Ca^{2+} in the lumen by the Ca^{2+}-ATPase and can release Ca^{2+} into the cytoplasm through the IP_3 and ryanodine release channels. Electroneutrality of the ER membrane during Ca^{2+} movement in either direction is maintained by the coupled transport of Cl^-. The results of the present study show that secretory vesicles contain many of the same transport pathways that have been shown to be present in the ER. Therefore, it is possible that the Ca^{2+} handling mechanisms are similar in the vesicle and the ER as well.

The present results would suggest a model whereby the secretory vesicles express the IP_3 and ryanodine receptors as the pathway for Ca^{2+} release. This assertion is based upon both the immunological localization of the channel proteins as well as the pharmacological manipulation of the vesicle's physiology. These release pathways are thought to be functional based on results showing alteration in fusion efficiency and cation dependent vesicle swelling in response to Ca^{2+} and Cl^- channel blockers. Therefore it is viewed that hormone stimulation of the pancreatic acinar cell would result in production of

IP_3, an initial release of Ca^{2+} from secretory vesicle stores, followed by the CICR response of the ryanodine Ca^{2+} release pathway also located on the secretory vesicle. The resultant decrease in intravesicular Ca^{2+} would in turn promote protein solubilization, osmotic swelling, and an increase in exocytotic fusion efficiency.

REFERENCES

Anderie, I. and F. Thevenod (1996) Evidence for involvement of a zymogen granule Na^+/H^+ exchanger in enzyme secretion from rat pancreatic acinar cells. J. Membrane Biol., **152**: 195-205.

Bahnson, T., S. Pandol, and V. Dionne (1993) Cyclic GMP modulates depletion-activated Ca^{2+} entry in pancreatic acinar cells. J. Biol. Chem., **268**: 10808-10812.

Beaufay, H., A. Amar-Costesec, and E. Feytmans (1974) Analytical study of microsomes and isolated subcellular membranes from rat liver. J. Cell. Biol., 61:188-200.

Berridge, M. (1993) Inositol trisphosphate and calcium signalling. Nature, **361**:315-325.

Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem., **256**:1604-1607.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of Microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., **72**:248-254.

Carew, M. and P. Thorn (1996) Identification of ClC-2-like chloride currents in pig pancreatic acinar cells. Eur. J. Physiol. **433**: 84-90.

Coronado, R., J. Morrissette, M. Sukhareva, and D. Vaughan (1994) Structure and function of ryanodine receptors. Am. J. Physiol., **266**: C1485-C1504.

DeLisle, R.C., I. Schulz, T. Tryakowski, W. Hasse, and U. Hopfer (1984) Isolation of stable pancreatic zymogen granules. Am. J. Physiol., **246**: G411-G41.

Edwardson, J. and Marciniak, S. (1995) Molecular mechanisms in exocytosis. J. Membrane Biol. **146**: 113-122.

Fernandez, J. M., M. Villalon, and P. Verdugo (1991) Reversible condensation of mast cell secretory products in vitro. Biophys. J., **59**: 1022-1027.

Galione, A., H. Lee, and W. Busa (1991) Ca^{2+} -induced Ca^{2+} release in sea urchin egg homogenates: Modulation by cyclic ADP-ribose. Science, **253**: 1143-1146.

Gasser, K.W., J. DiDomenico, and U. Hopfer (1988) Potassium transport by pancreatic and parotid zymogen granule membranes. Am. J. Physiol., **255**:C705-C711

Gasser, K. W., DiDomenico, J., and Hopfer, U. (1988) Secretagogues activate chloride transport pathways in pancreatic zymogen granules. Am. J. Physiol., **254**: G93-G99.

Gasser, K.W. and J. Holda (1993) ATP-sensitive potassium transport by pancreatic secretory granule membrane. Am. J. Physiol., **264**:G137-G142.

Gasser, K.W. and J. Holda (1994) The effect of phospholipase A_2 on chloride transport by pancreatic secretory granules. Biochim. Biophys. Acta, **1194**: 123-130.

Gasser, K.W. and U. Hopfer (1990) Chloride transport across the membrane of parotid secretory granules. Am. J. Physiol., **259**:C413-C420.

Gasser, K. W., and L.B. Kirschner (1987) The response of alkaline phosphatase to osmoregulatory changes in the trout, *Salmo goirdneri*. J. Comp. Physiol. B, **157**:469-475.

Gasser, K.W., S. Oh, H. McDonald, and C. Johnson (1997) Evidence for regulation of rat pancreatic zymogen granule K^+ transport and membrane fusion by rab3B. Biophys. J. **72**: A300.

Gerasimenko, O. V., J. Gerasimenko, P. Belan, and O. Petersen (1996) Inositol triphosphate and cyclic ADP-ribose-mediated release of Ca^{2+} from single isolated pancreatic zymogen granules. Cell., **84**: 473-480.

Gonzalez, A., J. Pariente, G. Salido, and P. Camello (1997) Intracellular pH and calcium signalling in rat pancreatic acinar cells. Eur. J. Physiol., **434**: 609-614.

Gorelick, F., and J. Jamieson (1994) The pancreatic acinar cell: stucture-function releationships. In: Physiology of the Gastrointestinal Tract, 3rd edition, L. Johsnon ed. Raven Press, New York, pp. 1353-1376.

Hoekstra, D., T. deBoer, K. Klappe, and J. Wilschut (1984) Fluorescence method for measuring the kinetics of fusion between biological membranes. Biochemistry, **23**: 5675-5681

Kasai, H. (1995) Pancreatic calcium waves and secretion. Ciba Foundation Symp., **188**: 104-120

Kern, H. (1993) Fine structure of the human exocrine pancreas. In: The Pancreas: Biology, Pathobiology, and Disease, 2nd. Edition, V.L. Go, ed. Raven Press, New York, pp. 9-19.

Kim, H., E. Jacobson, and M. Jacobson (1993) Synthesis and degradation of cyclic ADP-ribose by NAD glycohydrolases. Science, **261**: 1330-1334.

Kourie, J. I. (1997) ATP-sensitive voltage- and calcium-dependent chloride channels in sarcoplasmic reticulum vesicles from rabbit skeletal muscle. J. Membrane Biol., **157**: 39-51.

Kourie, J. I., P. Foster, and A. Dulhunty (1997) Inositol phosphates modify the kinetics of a small chloride channel in skeletal muscle sarcoplasmic reticulum. J. Membrane Biol., **157**: 147-158.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature, **227**:680-685.

Lee, M.G., X. Xu, W. Zeng, J. Diaz, R. Wojcikiewicz, T. Kuo, F. Wuytack, L. Racymaekers, and S. Muallem (1997) Polarized expression of Ca^{2+} channels in pancreatic and salivary gland cells., J. Biol. Chem. **272**: 15765-15770.

Maranto, A. R. (1994) Primary structure, ligand binding and localization of inositol 1,4,5-triphosphate receptors expressed in intestinal epithelium. J. Biol. Chem., **269**: 1222-1230.

Meissner, G. (1984) Adenine nucleotidestimulation of Ca2+-induced Ca2+ release in sarcoplasmic reticulum. J. Biol. Chem., **259**: 2365-2374.

McPherson, P. and K. Campbell (1993) The ryanodine receptor/Ca^{2+} release channel. J. Biol. Chem., **268**: 13765-13768.

Mikoshiba, K. (1997) The $InsP_3$ receptor and intracellular Ca^{2+} signaling. Current Opinion in Neurobiology, **7**: 339-345.

Mogami, H., K. Nakano, A. Tepikin, and O. Petersen (1997) Ca^{2+} flow via tunnels in polarized cells: Recharging of apical Ca^{2+} stores by focal Ca^{2+} entry through basal membrane patch. Cell., **88**: 49-55.

Moser, T., R. Chow, and E. Neher (1995) Swelling-induced catecholamine secretion recorded from single chromaffin cells. Eur. J. Physiol., **431**: 196-203.

Nathanson, M.H., M. Fallon, P. Padfield, and A. Maranto (1994) Localization of the type 3 inositol 1,4,5-trisphosphate receptor in the Ca^{2+} wave trigger zone of pancreatic acinar cells. J. Biol. Chem., **269**: 4693-4696.

Nicaise, G., K. Maggio, S. Thirion, M. Horoyan, and E. Keicher (1992) The calcium loading of secretory granules. A possible key event in stimulus-secretion coupling. J. Biol. Cell, **75**: 89-99.

Ohnishi, H., S. Ernst, N. Wys, M. McNiven, and J. Williams (1996) Rab3D localizes to zymogen granules in rat pancreatic acini and other exocrine glands. Am. J. Physiol., **271**: G531-G538.

Ozawa, T., and A. Nishiyama (1997) Characterization of ryanodine-sensitive Ca^{2+} release from microsomal vesicles of rat parotid acinar cells: Regulation by cyclic ADP-ribose. J. Membrane Biol., **156**: 231-239.

Peterson G. L. (1978) A simplified method for analysis of inorganic phosphate in the presence of interfering substances. Anal. Biochem., **84**:164-172.

Putney, J. W. Jr. (1997) Capacitative Calcium Entry., R.G. Landes Publ. Austin, TX pp. 210.

Rios, E., G. Pizarro, and E. Stefani (1992) charge movement and the nature of signal transduction in skeletal muscle excitation- contraction coupling. Ann. Rev. Physiol., **54**: 109-133.

Scarpa, A., and P. Graziotti (1973) Mechanisms for intracellular calcium regulation in the heart. I. Stopped-flow measurements of Ca^{2+} uptake by cardiac mitochondria. J. Gen. Physiol. **62**: 756-772

Shuttleworth, T. (1997) Intracellular Ca^{2+} signaling in secretory cells. J. Exp. Biol., **200**: 303-314.

Sorrentino, V. (1995) The ryanodine receptor family of intracellular calcium release channels. Advances in Pharmacology, **33**: 67-91.

Sottocasa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand (1967) An Electron transport system associated with the outer membrane of liver mitochondria. J. Cell Biol., **32**:415-438.

Stahl, A. (1996) Electrolyte transport by pancreatic secretory granules regulates exocytotic membrane fusion. Ph.D. Dissertation, Northern Illinois University.

Streb H., R. Irvine, M. Berridge, and I. Schulz (1983) Release of Ca^{2+} from a nonmitochondrial store in pancreatic cells by inositol -1,4,5-trisphosphate. Nature, **306**: 67-68.

Striggow, F. and B. Ehrlich (1996) Ligand-gated calcium channels inside and out. Current Opinion in Cell Biol., **8**: 490-495.

Subramanian, K. and T. Meyer (1997) Calcium-induced restructuring of nuclear envelope and endoplasmic reticulum calcium stores. Cell, **89**: 963-971.

Thevenod, F., K. Chathadi, B. Jiang, and B. Hopfer (1992) ATP-senstive K^+ conductance in pancreatic zymogen granules: block by glyburide and activation by diazoxide. J. Membrane Biol. **129**: 253-266.

Thorn, P., A. Lawrie, P. Smith, D. Gallacher, and O. Petersen (1993) Local and global cytosolic Ca^{2+} oscillations in exocrine cells evoked by agonists and inositol triphosphate. Cell, **74**: 661-668.

Thorn, P., O. Gerasimenko, and O. Petersen (1994) Cyclic ADP-ribose regulation of ryanodine receptors involved in agonist evoked cytosolic Ca^{2+} oscillations in pancreatic acinar cells. EMBO J., **13**: 2038-2043.

Titievsky, A.V., T. Takeo, A. Tepikin, and O. Petersen (1996) Decrease of acidity inside zymogen granules inhibits acetylcholine- or inositol triphosphate- evoked cytosolic Ca^{2+} spiking in pancreatic acinar cells. Eur. J. Physiol., **432**: 938-940.

Tsunoda, Y. (1993) Receptor -operated Ca^{2+} signaling and crosstalk in stimulus secretion coupling. Biochim. Biophys. Acta., **1154**:105-156.

Valdivia, H., J. Kaplan, G. Ellis-Davies, and W. Lederer (1997) Rapid adaptation of cardiac ryanodine receptors: Modulation by Mg^{2+} and phosphorylation. Science, **267**: 1997-2000.

Walensky, L., and S. Snyder (1995) Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. J. Cell Biol., **130**: 857-869.

Yule, D.I., S. Ernst, H. Ohnishi, and R. Wojcikiewicz (1997) Evidence that zymogen granules are not a physiologically relevant calcium pool. J. Biol. Chem., **272**: 9093-9098.

Zimmerberg, J. and J. Liu (1988) Ionic and permeability requirements for exocytosis in vitro in sea urchin eggs. J. Membrane Biol., **101**:199-207.